Antifungal activity of fermented dairy ingredients: Identification of antifungal compounds

Lucille Garnier, Marine Penland, Anne Thierry, Marie-Bernadette Maillard, Julien Jardin, Monika Coton, Marcia Leyva Salas, Emmanuel Coton, Florence Valence, Jérôme Mounier

Antifungal activity of fermented dairy ingredients: identification of antifungal compounds.

Lucille Garnier[1,2†], Marine Penland[1†], Anne Thierry[2], Marie-Bernadette Maillard[2], Julien Jardin[2], Coton Monika[1], Marcia Leyva Salas[1,2], Emmanuel Coton[1], Florence Valence[2] and Jérôme Mounier[1]*.

[1]Univ Brest, Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, F-29280 Plouzané, France

[2] STLO, Agrocampus Ouest, INRA, 35000 Rennes, France

[†]These authors equally contributed to the work

Lucille Garnier : lucille.garnier@univ-brest.fr, Marine Penland: marine.penland@univ-brest.fr, Anne Thierry: anne.thierry@inra.fr, Marie-Bernadette Maillard: marie-bernadette.maillard@inra.fr, Julien Jardin: julien.jardin@inra.fr, Monika Coton : monika.coton@univ-brest.fr; Marcia Leyva Salas : marcia.leyvasalas@univ-brest.fr, Emmanuel Coton : emmanuel.coton@univ-brest.fr; Florence Valence : florence.valence-bertel@inra.fr

*__Corresponding author__ : Jérôme Mounier

Laboratoire Universitaire de Biodiversité et Ecologie Microbienne,

Parvis Blaise-Pascal, Technopôle Brest-Iroise

29280 Plouzané, France

Tel : +33 (0)2.90.91.51.00

E-mail: Jerome.mounier@univ-brest.fr

Keywords: antifungal; fermentate; organic acids; fatty acids; volatile compounds

Abstract

Fungi are commonly identified as the cause for dairy food spoilage. This can lead to substantial economic losses for the dairy industry as well as consumer dissatisfaction. In this context, biopreservation of fermented dairy products using lactic acid bacteria, propionibacteria and fungi capable of producing a large range of antifungal metabolites is of major interest. In a previous study, extensive screening was performed *in vitro* and *in situ* to select 3 dairy fermentates (derived from *Propionibacterium jensenii* CIRM-BIA1774, *Lactobacillus rhamnosus* CIRM-BIA1952 and *Mucor lanceolatus* UBOCC-A-109193, respectively) with antifungal activity. The aim of the present study was to determine the main compounds responsible for this antifungal activity. Fifty-six known antifungal compounds as well as volatiles were targeted using different analytical methods (conventional LC and GC, GC-MS, LC-QToF). The most abundant antifungal compounds in *P. jensenii*-, *L. rhamnosus*- and *M. lanceolatus*-derived fermentates corresponded to propionic and acetic acids, lactic and acetic acids, and butyric acid, respectively. Many other antifungal compounds (organic acids, free fatty acids, volatile compounds) were identified but at lower levels. In addition, an untargeted approach using nano LC-MS/MS identified a 9-amino acid peptide derived from α_{s2}-casein in the *L. rhamnosus*-derived fermentate. This peptide inhibited *M. racemosus* and *R. mucilaginosa in vitro*. This study provides new insights on the molecules involved in antifungal activities of food-grade microorganism fermentates which could be used as antifungal ingredients in the dairy industry.

1. Introduction

Fungi can cause spoilage of dairy products. Indeed, due to their intrinsic characteristics (e.g. acidic pH and intermediate water activity, nutrient composition), dairy products constitute a favorable environment for yeast and mold growth (Rohm et al., 1992). Fungal spoilage leads to food waste and substantial economic losses, and can also impact brand image in the eye of the consumer (Pitt and Hocking, 2009). The most common fungi involved in dairy product spoilage belong to *Penicillium*, *Mucor* and *Cladosporium* genera for moulds and *Candida*, *Kluyveromyces* and *Yarrowia* for yeasts (Garnier et al., 2017; Pitt and Hocking, 2009).

Control of fungal spoilers in the dairy industry is therefore of utmost importance. Today, different preventive and control methods (also known as "hurdle technologies") are implemented and combined to prevent contamination during product manufacturing but also to inhibit or slow down growth of fungal spoilers. These methods include, for example, use of good manufacturing and hygiene practices, implementation of the hazard analysis and critical control points (HACCP) system, air filtration and packaging in aseptic conditions, heat treatment, refrigeration and salting/brining. In addition, chemical preservatives can be used and extend dairy product shelf-life with respect to relevant legislation (Garnier et al., 2017). However, regarding chemical preservatives, consumers are more frequently requesting preservative-free products. "Natural" preservation alternatives, such as biopreservation, are therefore increasingly used (Leyva Salas et al., 2017).

Lactic acid bacteria (LAB), propionibacteria and fungi are good candidates for biopreservation as they have a long history of safe use in human consumption. LAB are used in various dairy products such as yogurt, cheese or kefir (Corrieu and Luquet, 2008). As a consequence, some LAB, but also propionibacteria species, are used as bioprotective agents (as cultures or antifungal ingredients) in dairy products. Commercial antifungal solutions such as Holdbac (DuPont Danisco, Dangé, Saint Romain, France), FRESHQ (CHR Hansen,

Hovedstaden, Danemark) and Natamax (DuPont Danisco, Dangé, Saint Romain, France) protective cultures containing LAB species and/or propionibacteria, as well as antifungal ingredients such as MicroGARD, derived from Propionibacteria species, are currently available on the market (Leyva Salas et al. 2017). These microorganisms are able to produce a large spectrum of compounds such as organic acids, fatty acids (Sjögren et al., 2003), cyclic dipeptides (Ström et al., 2002) and proteinaceous compounds (Rizzello et al., 2011) that are potentially involved in antifungal activities, acting in synergy (Crowley et al., 2013; Leyva Salas et al., 2017). Concerning fungal biocontrol agents, they are mostly used in fruit preparations or fermented meat (Leyva Salas et al., 2017). Studies have shown their ability to produce antifungal compounds such as killer-toxins, organic acids or peptides (Acosta et al., 2009 ; Coda et al., 2013 ; Ignatova et al., 2015 ; Leyva Salas et al., 2017; Nally et al., 2015).

When it comes to dairy product biopreservation, only a few studies have dealt with the action mechanisms of bioprotective cultures (Crowley et al., 2013). In a recent study, Mieszkin et al. (2017) explored the action mechanisms of *Lactobacillus harbinensis* K.V9.3.1.Np against *Yarrowia lipolytica* in fermented milk. The observed fungistatic effect of the tested bioprotective culture involved different organic acids and led a decrease in intracellular pH and membrane depolarization. More recently, Leyva Salas et al. (2019), using different analytical tools targeting 56 known antifungal compounds and volatile compounds, highlighted the diversity of antifungal molecules produced by different bioprotective cultures in various dairy products.

In another recent study (Garnier et al., 2019), 3 ingredients corresponding to dairy microbial fermentates with antifungal activity were selected after *in vitro* screening. Their efficiency was further validated in real dairy products (*i.e.* after incorporation in sour cream and surface-spraying on semi-hard cheese). The aim of the present study was to investigate which compounds were responsible for the antifungal activity in the previously selected fermentates.

To do so, different analytical methods, namely conventional LC and GC, GC-MS, LC-QToF and nano-LC-MS/MS, were applied, to detect and quantify antifungal metabolites, including 56 organic and fatty acids (targeted approach), and volatile compounds and peptides (non-targeted approach).

2. Material and methods

2.1. Fermentate preparation

Fermentates with antifungal activity were selected from a previous study (Garnier et al., 2019). They were obtained from 2 fermented dairy media: a reconstituted 10% low heat milk supplemented with 45% anhydrous milk fat (for the *Lactobacillus rhamnosus* CIRM-BIA1952 and *Mucor lanceolatus* UBOCC-A-109119 strains) and an ultrafiltration permeate supplemented with 1% yeast extract (for the *Propionibacterium jensenii* CIRM-BIA1774 strain). They were kept as a lyophilisate for *P. jensenii* CIRM-BIA1774 and *L. rhamnosus* CIRM-BIA1952 and as a 0.45 µm filtered culture supernatant for *Mucor lanceolatus* UBOCC-A-109119, as previously described (Garnier et al., 2019). For each experiment, three biological replicates of each fermentate were analyzed.

2.2. Identification and quantification of potential antifungal compounds

2.2.1. Antifungal compounds identification by LC-QToF

2.2.1.1. Standard preparation

Thirty-one compounds with known antifungal activity were searched for as described previously (Brosnan et al. 2014; Le Lay et al., 2016) except that 5 additional molecules were added, namely, 4-di-tert-butylphenol, mevalonolactone, N-acetyl-D-glucosamine, phenyl acetate and ricinoleic acid (Leyva Salas et al., 2019). Standards were prepared by mixing all compounds at different concentrations. Individual stock solutions at 5 mg/mL were prepared

in water or acetonitrile, and mixed together to obtain a standard mix at 100 ppm in H_2O/ACN (90/10, v/v). A matrix-matched calibration curve was built by diluting the standard mix in a blank extract of acidified semi-skimmed milk (adjusted to pH 5 with lactic acid) at the following concentrations: 1 ppm, 5 ppm, 10 ppm, 30 ppm and 50 ppm. Standards were kept at -20 °C in amber vials.

2.2.1.2. Sample preparation

Compound extraction from fermentates (lyophilized or filtered supernatant) were performed as described by Brosnan et al. (2014) with slight modifications. First, 10 mL of either lyophilized fermentate resuspended in sterile water (1:10, w/v) or of supernatant were mixed with 4 g of $MgSO_4$, 1 g of NaCl and 10 mL ethyl acetate supplemented with 1 % of formic acid. The mixture was then vigorously shaken before centrifuging for 10 min at 8500 *g* at 4 °C. The organic phase was transferred into a dSPE tube (dispersive Solid Phase Extraction, Agilent technologies), vortexed and centrifuged for 10 min at 2600 *g*. The liquid phase was then recovered in a 15 mL tube, mixed with 100 µL of DMSO and evaporated under nitrogen gas. The remaining 100 µL were supplemented with 900 µl of H_2O/acetonitrile (90/10, v/v), filtered at 0.22 µm and stored in amber vials at -20 °C until analysis.

2.2.1.3. LC-QToF analysis and method validation

Detection and quantification were performed on a 1260 Infinity binary HPLC and a 6530 Accurate Mass LC-QToF LC/MS (Agilent Technologies) as described by Le Lay et al. (2016) and Leyva Salas et al. (2019). Compounds separation was done on a Zorbax Extend-C18 column (201 x 150 mm, 5 µ) equipped with a pre-column (2.1 x 12.5 mm, 5 µ) (Agilent technologies) and the mass spectrometer operated in negative electrospray ionization.

Analyses were performed under the conditions described by Le Lay et al. (2016) except that injected volumes were 10 and 50 µL. Standards and samples were injected in triplicate.

To validate the method, extraction recovery was determined on acidified semi-skimmed milk spiked with 10 ppm of each compound. Standard mix at 10 ppm in blank extract was injected 3 times a day for 5 consecutive days to assess the intra- and inter-day repeatability. For each compound, the limit of detection (LOD) and limit of quantification (LOQ) were determined based on the standard deviation of the analyte response and the standard deviation slope (ICH Harmonized Tripartite Guideline, 2005)

2.2.2. Antifungal organics acid quantification by HPLC

Nine mL of fermentate supernatant or 3 g of lyophilized fermentates dissolved in sterile water (1:2) were centrifuged for 30 min at 10 000 *g*. The liquid phase was half diluted in 5 mM sulfuric acid, vigorously mixed and stored overnight at -20°C. After thawing, samples were once again centrifuged at 10 000 *g* for 30 min, the upper phase recovered and filtered on 0.45 µm PTFE membrane. Analysis was performed as previously described (Leyva Salas et al., 2019) on a HPLC Dionex system (Sunnydale, CA, USA) equipped with an Aminex HPX-87H column (Biorad, Hercules, CA, USA), using 5 mM H_2SO4 as the mobile phase, and UV (210 nm) and refractometer detectors. Identification and quantification were achieved using standard solutions of the following organic acids at concentrations ranging from 0 to 1 mg/mL: acetic acid, benzoic acid, citric acid, lactic acid, propionic acid, succinic acid and 2-pyrrolidone-5-carboxylic acid.

2.2.3. Free fatty acid identification by gas chromatography

2.2.3.1. Standard preparation

Identification and quantification of free fatty acids, as described by Leyva Salas et al. (2019), was adapted from the method by Jong and Badings (1990). They were performed with different external standards, including acetic, propionic, butyric, valeric, isovaleric, caproic, enanthic, caprylic, pelargonic, capric, undecyclic, lauric, myristic, palmitic, margaric, stearic, oleic, linoleic and octadecadienoic acids, with concentrations ranging from 5 to 500 µg/g. An internal standard, to take into account the extraction yield, was used (0.5 mg/g of valeric acid (C5:0), tridecanoic acid (C13:0) and margaric acid (C17:0) diluted in 30 mL of heptane).

2.2.3.2. Fatty acid extraction

Free fatty acid extraction was performed with 1 g of lyophilized fermentate or 1 mL of supernatant, 3 g of anhydrous sodium sulfate (Na_2SO_4) and the mixture was ground. Then, 0.3 mL of H_2SO_4 at 2.5M, 1 mL of internal standards and 15 mL of ether/heptane (1:1) were added before centrifugation (3 min at 100 *g*). The organic layer was recovered with 100 mL of Na_2SO_4 to capture residual water. The pellet was resuspended in 15 mL of ether/heptane (1:1) and centrifuged (3 min at 100 *g*). This step was repeated 3 times by pooling the organic layer. Free fatty acid purification was performed with a Manifold, using a SPE 500 mg column (Phenomenex, UK) conditioned with 10 mL heptane. The organic layer was transferred onto the column and 10 mL heptane/2-propanol (3:2) were added for neutral lipids removal. Free fatty acids were eluted with 5 mL ether diethyl with 2% formic acid and transferred in an amber vial and stocked at -20°C until analysis. Samples were injected in triplicate.

2.2.3.3. Analysis of fatty acids by GC

Fatty acid analysis was performed by gas chromatography (Varian CP-3800) equipped with a flame ionization detector (FID) and a capillary column (BP21 25m x 0.53 mm, layer 0.5 µm,

JW Scientific, Folsom, USA) as previously described (Leyva Salas et al., 2019). Vector gas was dihydrogen at 9.7 mL/min. A temperature gradient was applied from 65°C to 240°C with a ratio of 10°C/min then 10 min at 240°C using direct injection. Quantification was performed using standard calibration curves with the Star Varian (version 5.3) software. A correction of possible free fatty acid losses during extraction was performed with internal standards using the following calculation:

$$Free\ fatty\ acid\ (\mu g) = \frac{([Calculated\ concentration\ of\ free\ fatty\ acid] * [Standard\ Y\ (\mu g])}{[Calculated\ concentration\ of\ standard\ Y]}$$

2.2.4. Volatile compounds identification by GC-MS

2.2.4.1. Preparation of standards for GC-MS analysis

Standard compounds were used to generate standard curves and to check the response of the HS-trap GC-MS system during the sample analyses. Two solutions of standard compounds were prepared: one with neutral volatiles and another with short-chain fatty acids. Neutral standard compounds included 4 esters (ethyl acetate, ethyl propanoate, ethyl butanoate and ethyl hexanoate), 2 aldehydes (3-methylbutanal and benzaldehyde), 2 ketones (2-heptanone, 2,3-butanedione), 1 sulphur compound (dimethyl disulfide) at concentrations ranging from 5 to 1200 ng/g and 1 amyl alcohol (3-methylbutanol) at concentrations ranging from 260 to 50,000 ng/g. For short-chain acids, the standard mix 46975-U (Supelco, Sigma-Aldrich, Saint-Louis, USA) containing acetic, propanoic, butanoic, 2-methylpropanoic, pentanoic, 3-methylbutanoic, hexanoic, 4-methylpentanoic and heptanoic acids at 10mM each was used to prepare standard solutions at concentrations ranging from 20 to 1000 ng/g. The pH of these standard acid solutions was adjusted to 6.35±0.15 with NaOH.

2.2.4.2. Extraction of volatile compound using headspace trap

Extraction of volatile compounds was performed using a Perkin Elmer Turbomatrix HS-40 trap automatic headspace sampler with trap enrichment. Fermentate aliquots (2.5 mL) were placed in 22 mL PerkinElmer vials with polytetrafluorethylene (PFTE)/silicone septa and the used extraction conditions were those described by Pogačić et al. (2015).

2.2.4.3. Analysis of volatile compounds using GC-MS

Volatiles were analyzed using a Clarus 680 gas chromatograph coupled to a Clarus 600T quadrupole mass spectrometer (PerkinElmer, Courtaboeuf, France) as described in Pogačić et al. (2015) and Leyva Salas et al. (2019). All samples were analyzed in the same GC-MS run. Standards were regularly injected to verify the absence of instrumental drift of the GC-MS system and blank samples (boiled deionized water) were also injected to check for the absence of carry-over.

Volatile compounds were identified as described in Pogačić et al. (2015), comparing their retention index and mass spectral data from the NIST 2008 Mass Spectral Library (Scientific Instrument Services, Ringoes, NJ, USA) with those of authentic standards purchased from Sigma Aldrich (St. Quentin Fallavier, France).

2.2.4.4. Data processing

As described by Pogačić et al. (2015), the PerkinElmer Turbomass software, version 5.4.2.1617, was used to perform data pre-processing. After conversion of the GC-MS raw data files to netCDF format with Data Bridge (Perkin Elmer, Waltham, Massachusetts, USA), raw data were converted to time- and mass-aligned chromatographic peak areas using the open source XCMS package implemented with the R statistical language (Smith et al., 2006).

A heatmap was generated after mean-centered normalization by R packages of pheatmap v. 1.0.12 (Kolde, 2019).

2.2.5. Identification of potential antifungal peptides

2.2.5.1. Identification of peptides by nano-LC-MS/MS

Mass spectrometry (MS) analysis was adapted from that described by Nyemb-Diop et al. (2016), using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, San Jose, USA) equipped with a nanoelectrospray ion source. Briefly, samples were first concentrated using a pepMap100 µ-precolumn (C18 column, 300 µm i.d. × 5 mm length, 5 µm particle size, 100 Å pore size; Dionex, Amsterdam, The Netherlands), before peptide separation was performed on a PepMap RSLC column (C18 column, 75 µm i.d. × 150 mm length, 3 µm particle size, 100 Å pore size; Dionex); column temperature was maintained at 35°C along peptide separation that was performed at a flow rate of 0.3 $\mu L \cdot min^{-1}$ using solvent A (2% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water) and solvent B (95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) TFA in deionized water). The elution gradient was as follows: a first rise from 5 to 35% solvent B over 35 min, followed by a second rise from 35 to 85% solvent B over 2 min. Eluted peptides were directly electrosprayed into the mass spectrometer operating in positive ion mode with a voltage of 1.8 kV. The mass spectra were recorded in full MS mode using the m/z range 250–2000. The resolution of the mass analyzer for a *m/z* of 200 a.m.u (atomic mass unit) was set to 70,000 in the acquisition method. For each scan, the ten most intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution of 17,500 at *m/z* of 200 a.m.u and the parent ion was subsequently excluded from MS/MS fragmentation for 15 s. The instrument was externally calibrated according to the supplier's instructions. Peptides were identified from the MS/MS spectra using X!Tandem pipeline software

(Langella et al., 2017) The peptide identification database was an in-house database composed of major milk and egg proteins derived from www.uniprot.org (207 proteins in total). Database search parameters were specified as follows: a non-specific enzyme cleavage was selected; a 0.05 Da mass error was allowed for fragment ions while 10 ppm mass error was allowed for parent ions. The phosphorylation of serine and threonine were selected as variable modifications as well as the oxidation of methionine residues. For each identified peptide, a minimum score corresponding to an e-value below 0.05 was considered to be a prerequisite for valid peptide identification. The peptide false discovery rate was calculated by the software to be less than 0.5% using these parameters.

2.2.5.2. Bioinformatic analysis to identify potential antifungal peptides

Peptides (n=1236) described as possessing an antifungal activity were selected from the following database: ADP (Wang et al., 2004), BIOPEP (Minkiewicz et al., 2008), CAMP (Waghu et al., 2014), EROP (Zamyatnin et al., 2006), MilkAMP (Théolier et al., 2014) and PeptideDB. Then, a Blast between these peptides and those identified by nano-LCMS/MS in the 3 selected fermentates and in the 2 original dairy substrates (non-fermented) was performed to obtain a list of 61 peptides found in fermentates and/or in dairy substrates. After elimination of peptides present in the dairy substrates, a short list of 16 potential antifungal peptides was obtained.

2.2.5.3 Antifungal assay of peptides isolated from lyophilized cultures and supernatant

Sixteen peptides with potential antifungal activity and previously identified by nano-LC-QToF in the selected fermentates were neosynthetized (Biomatik, Ontario, Canada). Their individual antifungal activity against *M. racemosus* UBOCC-A-109155 and *R. mucilaginosa* UBOCC-A-216004 was assessed using an agar well diffusion method. Briefly, the targeted

fungi were included at a final concentration of $1x10^4$ spores or cells per mL in potato dextrose broth supplemented with 1% agar and the mix poured into 90 mm Petri dishes. Once solidified, six 9-mm-wells were cut per plate and filled with 100 µL of the peptide solutions prepared as follows: each of the tested peptides was dissolved in sterile distilled water to obtain a 5 mg/mL stock solution and diluted to 2, 1, 0.5 and 0.25 mg/mL. Agar plates were then incubated at 25 °C and the potential antifungal activity evaluated daily for 7 days. When applicable, diameters of inhibition zones were measured.

2.3 Statistical analyses

Statistical analyses were performed with the Statgraphics Plus software (Statpoint Technologies Inc., Herdon, VA, USA). One-way analysis of variance (ANOVA) was carried out to detect significant differences among means. A Fisher's least significant difference test was applied to compare the mean values.

3. Results

3.1. Identification and quantification of potential antifungal compounds

3.1.1. Organic and fatty acids

Overall, 31, 32 and 22 compounds among the 56 targeted organic and fatty acids were detected in the *P. jensenii* CIRM-BIA1774, *L. rhamnosus* CIRM-BIA1952 and *M. lanceolatus* UBOCC-A-109119 fermentates, respectively. Concerning organic acids, 11 of them were systematically detected in all 3 fermentates (Table 1). For bacterial fermentates, 3 organic acids (*i.e.*, propionic, citric and acetic acids), were quantified at concentrations above 10 mg/g in the *P. jensenii* fementate while only 2 (lactic and citric acids) were present above this level in the *L. rhamnosus* fermentate. In addition, 15 molecules were detected at concentrations comprised between the LOQ values and 1 mg/g in the *L. rhamnosus* fermentate versus 10 in the *P. jensenii* fermentate. Other detected molecules could not be

quantified as their values were below the LOQ. Concerning the *M. lanceolatus* fermentate, the 13 detected organic acids never exceeded 1 mg/mL in concentration except for citric acid. Lactic acid was present at a high concentration (86.018±3.05 mg/g fermentate) in the *L. rhamnosus* fermentate but absent in both the *P. jensenii* and *M. lanceolatus* fermentates. As expected, propionic acid was only quantified at high levels in the *P. jensenii* fermentate (59.94 ± 21.28 mg/g). Acetic acid concentrations were 16.893 ± 5.676 mg/g in the *P. jensenii* culture, 2.57 ± 0.62 mg/g (approximately 6-fold less) in that of *L. rhamnosus* and 0.104 ± 0.002 mg/g in the *M. lanceolatus* fermentate. For several compounds, including 2-pyrrolidone-5-carboxylic, (S)-2-hydroxy-4-methylpentanoic acid, hydroxyphenyllactic, phenyllactic acid and mevalonolactone, the highest concentrations were observed in the *P. jensenii* fermentate. Noteworthy, *L. rhamnosus* fermentate contained relatively high concentrations of succinic acid, 5-oxopyrrolidine-2-carboxylic acid, mevalonolactone, (S)-2-hydroxy-4-methylpentanoic acid and benzoic acid.

Concerning free fatty acids, 19 were detected in the different fermentates (Table 1). Among them, 12 compounds (butyric, caproic, hydroxyisocaproic, nonanoic, capric, lauric, myristic, pentadecanoic, palmitic, palmitoleic, stearic and oleic acids) were systematically found in the 3 fermentates at various concentrations (Table 1). Among these 12 compounds, 1 (heptanoic acid) and 3 (linoleic, nonadecanoic and arachidic acids) compounds were only found in the *L. rhamnosus* or *P. jensenii* fermentate, respectively, but concentrations were extremely low (< 2 µg/g). In addition, 7 compounds (caproic, nonanoic, capric, lauric, pentadecanoic, palmitoleic and oleic acids) showed concentrations below 10 µg/g or µg/mL. Among the 5 remaining compounds identified in all fermentates, quantitative differences were observed (Table 1). Myristic, palmitic and stearic acids were found at intermediate concentrations, *i.e.* ranging from >10 µg/mL myristic acid in the *M. lanceolatus* UBOCC-A-109193 fermentate to ~50 µg/g palmitic acid in *L. rhamnosus* CIRM-BIA1952. Finally, butyric acid was the most

abundant free fatty acid in the *M. lanceolatus* UBOCC-A-109193 fermentate (444.7±0.94 µg/mL) while hydroxyisocaproic acid was found in *P. jensenii* CIRM-BIA1774 and *L. rhamnosus* CIRM-BIA1952 fermentates with concentrations of 537±30 and 222±8 µg/g, respectively (Table 1).

3.1.2. Identification of volatile compounds by head-space trap GC-MS

An untargeted approach was used to search for potential antifungal volatile compounds in fermentates using head-space trap coupled to gas chromatography. Thirty-six volatile compounds were detected in the analysed fermentates (Figure 1). Among them, 15, 11 and 10 volatile compounds (including propionate and acetate, and butyrate which were accurately quantified using HPLC and GC-FID, respectively) were detected with the highest relative abundance in *P. jensenii* CIRM-BIA1774, *L. rhamnosus* CIRM-BIA1952 and *M. lanceolatus* UBOCC-A-119109 fermentates, respectively (Figure 1). As shown in Figure 1, the *P. jensenii* CIRM-BIA1774 fermentate contained, in addition to propionate and acetate, 20 to 1792 times higher relative abundances of propanoate esters (i.e. butyl propanoate, ethyl propanoate, 2-methyl propanoate and propyl propanoate) and acetate esters (i.e. ethyl acetate, butyl acetate and butanol- 2-methyl-acetate) in comparison to the other fermentates. In contrast, the *L. rhamnosus* CIRM-BIA1952 fermentate was characterized by high relative abundances of furans (2-ethyl furan, 2-n-butyl furan, 2-pentyl furan and 2-furamethanol) and several aldehydes (heptanal, octanal, nonanal) and ketones (acetoine, diacetyl, 2-nonanone and 2-undecanone). A different profile was observed for the *M. lanceolatus* fermentate with high relative abundances of butyrate, esters (ethyl butyrate and ethyl hexanoate), aldehydes (benzaldehyde and 4-methyl benzaldehyde), ketones (2-pentanone, 2, 3-pentanedione and diacetyl) and one alcohol (3-methyl 1-butanol).

3.1.3. Identification of potential antifungal peptides

An untargeted approach was applied to search for potential antifungal peptides present in the different fermentates. Regarding the nano-LC-MS/MS analysis, a total of 1040 peptides were identified in the 3 tested fermentates. Among these peptides, 22, 253 and 853 were identified in *P. jensenii* CIRM-BIA1774, *L. rhamnosus* CIRM-BIA1952 and *M. lanceolatus* UBOCC-A-103193 fermentates, respectively. In addition, *P. jensenii* and *L. rhamnosus* fermentates shared 3 and 85 peptides with the *M. lanceolatus* fermentate, respectively. There was no common peptide between *P. jensenii* and *L. rhamnosus* fermentates. After a Blast search against a list of 1236 known antifungal peptides, 16 peptides were neosynthesized for *in vitro* antifungal assays (Table 2). They had E-values ranging from $1.6x10^{-2}$ to $2x10^{-6}$, identities between 47 and 100% and relative abundances between $1.7x10^{-2}$ and $8.9x10^{-8}$. They corresponded to α_{s2} and κ-casein hydrolysates..

3.1.4. Antifungal activity of identified peptides

Out of the 16 peptides evaluated for antifungal activities by the agar diffusion method against *M. racemosus* and *R. mucilaginosa*, only one (pepa4c177 detected in *L. rhamnosus* fermentate) showed significant activity (Figure 2). This peptide contained 9 amino acids and was derived from α_{s2}-casein f(165-203) (Table 2). This peptide inhibited both *M. racemosus* and *R. mucilaginosa* at the highest tested concentrations (2.5 and 5 mg/mL) while only *M. racemosus* was inhibited at 1 mg/mL (Figure 2). After 7 days at 25°C, the peptide remained active against the two tested fungi, with inhibition zones of 2 and 3 mm in diameter around wells containing 2 and 5 mg/mL, respectively.

4. Discussion

In this study, 3 fermentates obtained from *L. rhamnosus*, *P. jensenii* and *M. lanceolatus*, previously shown to exhibit promising antifungal activities in dairy products (Garnier et al., 2019), were studied using a combination of analytical methods to identify the antifungal compounds involved in their activity. A large variety of organic and fatty acids, volatile compounds and one antifungal peptide was evidenced providing new insights about the types of molecules involved in the antifungal activities of microorganisms used for biopreservation.

As expected, lactic and acetic acids were the most abundant fermentation products for *L. rhamnosus* while propionic and acetic acids were linked to *P. jensenii*. Their respective and/or combined antifungal activities are well-known and have been reported many times in the literature (Bian et al., 2016; Gerez et al., 2010; Lind et al., 2007). It is worth mentioning that lactic acid *per se* is not considered as a compound with antifungal activity, given its relatively low pKa (3.9) and the pH encountered in dairy products. Nevertheless, Dagnas et al. (2015) showed that, in combination with acetic acid, both acted in synergy. In contrast to lactic acid, acetic and propionic acids have higher pKa values (pKa =4.75 and 4.87, respectively). Given their high concentrations in the bacterial fermentates, they likely contribute to the observed antifungal activity. Organic acid concentrations in the *M. lanceoalatus* fermentate were substantially inferior to the other two, except for citric acid that was quantified at high levels in all fermentates. It is also naturally present in milk. Although the antifungal activity of this acid has been reported, its efficiency is limited when compared to other organics acids such as acetate and benzoate (Hassan et al., 2015; Shokri, 2011). However, it also acts as a precursor for other compounds such as diacetyl which is well known for its antifungal activity as discussed below.

The *P. jensenii* fermentate, and to a lesser extent the *L. rhamnosus* one, contained numerous hydroxyled compounds such as 3-(4-hydroxyphenyl) propionic acid, (S)-(-)-2-hydroxyisocaproic acid and DL-hydroxyphenyllactic acid. The hydroxyl group improves acid

417 bioactivity by enhancing viscosity and reactivity as compared to their non-hydroxylated
418 counterpart and by providing an easier partition into the membrane (Pohl et al., 2011). Honoré
419 et al. (2016) showed that these compounds mostly contributed to the antifungal activity of
420 *Lactobacillus paracasei* DGCC 2132. It has also been reported that decanoic acid derived
421 hydroxylated acids inhibited *R. mucilaginosa* growth at concentrations as low as 10 µg/mL
422 (Sjögren et al., 2003). This compound could therefore contribute to the antifungal effect of the
423 *P. jensenii* fermentate. Phenyllactic acid, which was quantified at concentrations of 0.596
424 mg/g in the *P. jensenii* fermentate, has also been shown to be an efficient antifungal
425 compound (Lavermicocca et al., 2003).
426 Free fatty acids were also searched for and quantified. Concerning *L. rhamnosus* and *P.*
427 *jensenii* fermentates, concentrations were low (<0.06 mg/g) for all identified free fatty acids,
428 except for (S)-(-)-2-hydroxyisocaproic acid. Low concentrations were also observed in the *M.*
429 *lanceolatus* fermentate except for butyric acid which had a concentration of 0.537 mg/mL.
430 Butyric acid has been shown to exhibit antifungal activity (Pohl et al., 2011) and may play a
431 significant role in the observed *M. lanceolatus* fermentate antifungal activity. Its presence in
432 high concentration likely results from *M. lanceolatus* lipolytic activity.
433 Because volatile compounds may possess antifungal activity, as recently shown by Aunsbjerg
434 et al. (2015) for diacetyl, they were also analysed in the different fermentates. Diacetyl was
435 present in all 3 fermentates but the highest prevalence was observed in *L. rhamnosus* and *M.*
436 *racemosus* fermentates. This compound was shown to play a non-negligible role in the
437 antifungal activity of *L. paracasei* DGCC 2132 against two *Penicillium* spp. strains
438 (Aunsbjerg et al., 2015). Similarly, ethyl acetate was found in all fermentates and was
439 previously shown to be the main compound responsible of the antifungal activity of
440 *Wickerhanomyces anomalus* in sourdough bread (Coda et al., 2011). More recently, 2-methyl
441 propanoate, present at a high relative abundance in the *P. jensenii* fermentate, showed

increased antifungal activity against *Fusarium culmorum* and *Cochliobolus sativus* (Kaddes et al., 2019). However, to our best knowledge, the antifungal activity of most of the identified volatile compounds is not known. Consequently, it could be of interest to test their potential antifungal activity, alone or in combinations and, if active, to accurately quantify them.

Peptides can also be involved in the antifungal activity of biocontrol agents (Leyva Salas et al., 2017), therefore, an untargeted approach was used to identify such compounds. One basic peptide, pepa4c177 (RLNFLKKIS), from α_{s2}-casein f(165-203) and identified in the *L. rhamnosus* CIRM-BIA1952 fermentate, showed clear *in vitro* antifungal activity against *R. mucilaginosa* and *M. racemosus*, during the seven day evaluation at 2 and 1 mg/mL, respectively. The C-terminal region of this peptide is undoubtedly essential for its antifungal effect since the pepa4b61, pepa4c101 and pepa4b79 peptides, whose sequences partially overlapped with pepa4c177, turned out to be inactive. It would be necessary to quantify pepa4c177 in the fermentate to define whether it takes part,or not, in the observed *in situ* antifungal activity. Noteworthy, this antifungal peptide is not *de novo* synthesized by *L. rhamnosus* but results from its proteolytic activities. In most studies on antifungal LAB, cyclic dipeptides are the main peptides related to the observed antifungal activity. Their production by *Lactobacillus* species has already been reported (Magnusson et al., 2003; Ström et al., 2002). A MIC of 20 mg/mL was determined for *L. plantarum* cyclo(L-Phe-L-Pro) against *Aspergillus fumigatus* and *Penicillium roqueforti* (Ström et al., 2002), which is 10 times higher than that observed for pepa4c177. On the other hand, cyclo(L-Pro-D-Leu) produced by a *Bacillus cereus* subsp. *thuringiensis* strain had a MIC of 8 µg/mL against *Aspergillus flavus* (Nishanth Kumar et al., 2013). It would also be interesting to further investigate the action spectrum and MIC of the pepa4c177 peptide as it is a novel peptide described for the first time in the present study. Noteworthy, *L. rhamnosus* CIRM-BIA1952 antifungal activity was previously evaluated as an adjunct bioprotective culture in different

dairy product models (Leyva-Salas et al. 2018). Despite it showed, to some extent, a slight antifungal activity in a yogurt model, its activity was far less pronounced than for other tested *L. rhamnosus* strains (Leyva-Salas et al. 2018).

Overall, more than fifty compounds were identified in the 3 fermentates. It is reasonable to assume that they were not equally involved in the antifungal activity observed against *R. mucilaginosa* and *M. racemosus*. Moreover, synergistic and/or additive effects between compounds have been reported and make it more complex to understand their mechanism of action (Crowley et al., 2013; Suomalainen and Mäyrä-Makinen, 1999). To clarify this aspect, MIC determinations for each compound identified as well as their MIC in a mixture against the two fungi could be conducted.

The results reported in this study provide new insights to better understand the antifungal activities of bacterial and fungal fermentates. Further work will be necessary to precisely identify which molecules are the main actors involved in the observed antifungal activities of the studied fermentates, and how these molecules act and affect fungal cell physiology at a cellular level.

Acknowledgements

We are grateful to Regional Councils of Brittany (grant no. 13008651) and Pays de la Loire (grant no. 2014-07081) for their financial support and INRA for scientific coordination (J. Leonil) through the interregional project PROFIL, managed by BBA industrial association.

References

Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, Asensio, M.A., 2009. Selection of antifungal protein-producing molds from dry-cured meat products. Int. J. Food Microbiol. 135, 39-46.

492 Aunsbjerg, S.D., Honoré, A.H., Marcussen, J., Ebrahimi, P., Vogensen, F.K., Benfeldt, C.,
493 Skov, T., Knøchel, S., 2015. Contribution of volatiles to the antifungal effect of *Lactobacillus*
494 *paracasei* in defined medium and yogurt. Int. J. Food Microbiol. 194, 46–53.

495 Bian, X., Muhammad, Z., Evivie, S. E., Luo, G.-W., Xu, M., Huo, G.-C., 2016. Screening of
496 antifungal potentials of *Lactobacillus helveticus* KLDS 1.8701 against spoilage
497 microorganism and their effects on physicochemical properties and shelf life of fermented
498 soybean milk during preservation. Food Control 66, 183–189.

499 Brosnan, B., Coffey, A., Arendt, E.K., Furey, A., 2014. The QuEChERS approach in a novel
500 application for the identification of antifungal compounds produced by lactic acid bacteria
501 cultures. Talanta 129, 364–373.

502 Coda, R., Cassone, A., Rizzello, C.G., Nionelli, L., Cardinali, G., Gobbetti, M., 2011.
503 Antifungal activity of *Wickerhamomyces anomalus* and *Lactobacillus plantarum* during
504 sourdough fermentation: identification of novel compounds and long-term effect during
505 storage of wheat bread. Appl. Environ. Microbiol. 77, 3484–92.

506 Coda, R., Rizzello, C.G., Di Cagno, R., Trani, A., Cardinali, G., Gobbetti, M., 2013.
507 Antifungal activity of *Meyerozyma guilliermondii*: Identification of active compounds
508 synthesized during dough fermentation and their effect on long-term storage of wheat bread.
509 Food Microbiol. 33, 243-251.

510 Corrieu, G., Luquet, F.-M., 2008. Bactéries lactiques: de la génétique aux ferments. Ed.
511 Lavoisier/TEC&DOC, Paris, France.

512 Crowley, S., Mahony, J., van Sinderen, D. 2013. Current perspectives on antifungal lactic
513 acid bacteria as natural bio-preservatives. Trends Food Sci. Tech. 33, 93–109.

514 Dagnas, S., Gauvry, E., Onno, B., Membré, J.-M., 2015. Quantifying effect of lactic, acetic,
515 and propionic acids on growth of molds isolated from spoiled bakery products. J. Food Prot.
516 78, 1689–1698.
517 Garnier, L., Mounier J., Lê, S., Pawtowski, A., Pinon, N., Camier, B., Chatel, M., Garric G.,
518 Coton, E., Valence, F., 2019. Development of antifungal ingredients for dairy products: from
519 *in vitro* screening to pilot scale application. Food Microbiol. 81, 97-107.

520 Garnier, L., Valence, F., Mounier J., 2017. Diversity and control of spoilage fungi in dairy
521 products: an update. Microorganisms 5, 42.

522 Gerez, C.L., Carbajo, M.S., Rollán, G., Torres Leal, G., Font de Valdez, G., 2010. Inhibition
523 of Citrus Fungal Pathogens by Using Lactic Acid Bacteria. J. Food Sci. 75, M354–M359.

524 Hassan, R., El-Kadi, S., Sand, M., 2015. Effect of some organic acids on some fungal growth
525 and their toxins production. Int. J. Adv. Biol. 2, 1-11.

526 Honoré, A.H., Aunsbjerg, S.D., Ebrahimi, P., Thorsen, M., Benfeldt, C., Knøchel, S., Skov,
527 T., 2016. Metabolic footprinting for investigation of antifungal properties of *Lactobacillus*
528 *paracasei*. Anal. Bioanal. Chem. 408, 83–96.

529 ICH, 2005. Q2 (R1), "Validation of analytical procedures: text and methodology", ICH
530 Harmonised Tripartite Guideline. International Conference on Harmonisation of Technical
531 Requirements for Registration of Pharmaceuticals for Human Use, Chicago, USA, 2005.

532
533 Ignatova, L.V., Brazhnnikova, Y.V., berzhanova, R.Z., Mukasheval, T.D., 2015. Plant
534 growth-promoting and antifungal activity of yeasts from dark chesnut soil. Microbial Res.
535 175, 78-83.

536 Jong, C., Badings, H.T., 1990. Determination of free fatty acids in milk and cheese
537 procedures for extraction, clean up, and capillary gas chromatographic analysis. J. High Res.
538 Chromatog. 13, 94-98.
539 Kaddes, A., Fauconnier, M.-L., Sassi, K., Nasraoui, B., Jijakli, M.H., 2019. Antifungal
540 properties of two volatile organic compounds on barley pathogens and introduction to their
541 mechanism of action. Int. J. Environ. Res. Public Health 16, 2866.

542 Kolde R. 2019. Package 'pheatmap'. Version 1.0.12. Available at https://cran.r-
543 project.org/web/packages/pheatmap/index.html (accessed 5th of July 2019).
544 Langella, O., Valot, B., Balliau, T., Blein-Nicolas, M., Bonhomme, L., Zivy, M., 2017.
545 X!TandemPipeline: A tool to manage sequence redundancy for protein inference and
546 phosphosite identification, J. Prot. Res. 16, 494–503.

547 Lavermicocca, P., Valerio, F., Visconti, A., 2003. Antifungal activity of phenyllactic acid
548 against molds isolated from bakery products. Appl. Environ. Microbiol. 69, 634-640.
549
550 Le Lay, C., Mounier, J., Vasseur, V., Weill, A., Le Blay, G., Barbier, G., Coton, E., 2016.
551 *In vitro* and *in situ* screening of lactic acid bacteria and propionibacteria antifungal activities
552 against bakery product spoilage molds. Food Contr. 60, 247–255.

553 Leyva Salas, M., Mounier, J., Valence, F., Coton, M., Thierry, A., Coton, E., 2017.
554 Antifungal microbial agents for food biopreservation - a review. Microorganisms 5, 37.

555 Leyva Salas, M., Thierry, A., Lemaître, M., Garric, G., Harel-Oger, M., Chatel, M., Lê, S.,
556 Mounier, J., Valence, F., Coton, E., 2018. Antifungal activity of lactic acid bacteria
557 combinations in dairy mimicking models and their potential as bioprotective cultures in pilot
558 scale applications. Front Microbiol. 9, 1787.

559 Leyva Salas, M., Mounier J., Maillard M.-B., Valence F., Coton E., Thierry A., 2019.
560 Identification and quantification of natural compounds produced by antifungal bioprotective
561 cultures in dairy products. Food Chem. 301, 125-260.

562 Lind, H., Sjögren, J., Gohil, S., Kenne, L., Schnürer, J., Broberg, A. 2007. Antifungal
563 compounds from cultures of dairy propionibacteria type strains. FEMS Microbiol. Lett. 271,
564 310–315.

565 Magnusson, J., Ström, K., Roos, S., Sjögren, J., Schnürer, J., 2003. Broad and complex
566 antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol.
567 Lett. 219, 129–135.

568 Mieszkin, S., Hymery, N., Debaets, S., Coton, E., Le Blay, E., Valence, F., Mounier, J., 2017.
569 Action mechanisms involved in the bioprotective effect of *Lactobacillus harbinensis*
570 K.V9.3.1.Np against *Yarrowia lipolytica* in fermented milk. Int. J. Food Microbiol. 248, 47-
571 55.

572 Minkiewicz, P. Dziuba, J., Iwaniak, A., Dziuba, M., Darewicz, M., 2008. BIOPEP database
573 and other programs for processing bioactive peptide sequences. J. AOAC Int. 91, 965-980.

574 Nally, M.C., Pesce, V.M., Maturano, Y.P., Rodriguez Assaf, L.A., Toro, M.E., Castellanos de
575 Figueroa, L.I., Vazquez, F., 2015. Antifungal modes of action of *Saccharomyces* and other
576 biocontrol yeasts against fungi isolated from sour and grey rots. Int. J. Food Microbiol. 204,
577 91-100.

578 Nishanth Kumar, S., Mohandas, C., Nambisan, B., 2013. Purification of an antifungal
579 compound, cyclo(l-Pro-d-Leu) for cereals produced by *Bacillus cereus* subsp. *thuringiensis*
580 associated with entomopathogenic nematode. Microbiol. Res. 168, 278-288.
581

582 Nyemb-Diop, K., Causeur, D., Jardin, J., Briard-Bion, V., Guérin-Dubiard, C., Rutherfurd,
583 S.M., Dupont, D., Nau, F., 2016. Investigating the impact of egg white gel structure on peptde
584 kinetics profil during *in vitro* digestion. Food Res. Int. 88, 302-309.
585 Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage. Springer Science Business Media,
586 Berlin, Germany.

587 Pogačić, T., Maillars, M-B., Leclerc, A., Hervé, C., Chuat, V., Yee, A.L., Valence, F., 2015.
588 A methodological approach to screen diverse cheese-related bacteria for their ability to
589 produce aroma compounds. Food Microbiol. 46, 145-153.

590 Pohl, C.H., Kock J.L.F., Thibane, V.S., 2011. Antifungal free fatty acids: A Review.
591 In: Méndez-Vilas, A. (Ed.), Science against microbial pathogens: communicating current
592 research and technological advances. Formatex Research Center, Badajoz, Spain, pp. 61-71

593 Rizzello, C.G., Cassone, A., Coda, R., Gobbetti, M., 2011. Antifungal activity of sourdough
594 fermented wheat germ used as an ingredient for bread making. Food Chem. 127, 952–959.

595 Rohm, H., Eliskases-Lechner, F., Bräuer, M., 1992. Diversity of yeasts in selected dairy
596 products. J. Appl. Microbiol. 72, 370–376.
597 Shokri, H., 2011. Evaluation of inhibitory effects of citric and tartaric acids and their
598 combination on the growth of *Trichophyton mentagrophytes*, *Aspergillus fumigatus*, *Candida*
599 *albicans* and *Malassezia furfur*. Comp. Clinic. Pathol. 20, 543-545.

600 Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J., Kenne, L., 2003. Antifungal 3-hydroxy
601 fatty acids from *Lactobacillus plantarum* MiLAB 14. Appl. Environ. Microbiol. 69, 7554–
602 7557.

603 Smith, C.A., Want, E.J., O'Maille, G., Abagyan, R., Siuzdak, G., 2006. XCMS: processing
604 mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching,
605 and identification. Anal. Chem. 78, 779-787

606 Ström, K., Sjögren, J., Broberg, A., Schnürer, J., 2002. *Lactobacillus plantarum* MiLAB 393
607 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-pro) and cyclo (L-Phetrans-4-OH-L-
608 pro) and 3-phenyllactic acid. Appl. Environ. Microbiol. 68, 4322–4327.
609 Suomalainen, T.H., Mäyrä-Makinen, A.M., 1999. Propionic acid bacteria as protective
610 cultures in fermented milks and breads. Lait 79, 165–174.

611 Théolier, J., Fliss, I., Jean, J., Hammami, R., 2014. MilkAMP: a comprehensive database of
612 antimicrobial peptides of dairy origin. Dairy Sci. Technol. 94, 181-193.

613 Waghu, F.H., Gopi, L., Barai, R.S., Ramteke, P., Nizami, B., Idicula-Thomas, S., 2014.
614 CAMP: Collection of sequences and structures of antimicrobial peptides. Nucleic acids Res.
615 42, 1154-1158.

616 Wang, G., Li, X., Wang, Z., 2004. ADP: the antimicrobial peptide database as a tool for
617 research and education. Nucleic Acids Res. 32, 590-592.

618 Zamyatnin, A.A., Borchikov, A.S., Vladimirov, M.G., Voronina, O.L., 2006. The EROP-
619 Moscow oligopeptide database. Nucleic Acids Res. 34, 261-266.

620

621 **Table 1**. **Organic and fatty acids with known antifungal activity detected and/or quantified in antifungal fermentates.**

		Fermentate		
Compound (common name)	Analysis method*	*P. jensenii* CIRM-BIA1774 (µg/g)	*L. rhamnosus* CIRM-BIA1952 (µg/g)	*M. lanceolatus* UBOCC-A-109193 (µg/mL)
Organic acids				
Ethanoic acid (acetic acid)	1	**$1.69x10^4 \pm 0.57x10^4$ [a]**	$2.57x10^3 \pm 0.62x10^3$ [b]	$1.04x10^2 \pm 2$ [c]
Propanoic acid (propionic acid)	1	**$5.99x10^4 \pm 2.128x10^4$ [a]**	$9.99x10^2 \pm 8.9x10^2$ [b]	-
2-Hydroxypropanoic acid (DL-lactic acid)	1	-	**$8.60x10^4 \pm 0.3x10^4$**	-
Butanedioic acid (succinic acid)	1	$4.85x10^3 \pm 2.78x10^3$ [a]	**$5.22x10^3 \pm 0.5x10^3$ [a]**	99 ± 24 [b]
5-Oxopyrrolidine-2-carboxylic acid (2-pyrrolidone-5-carboxylic acid)	2	**$2.23x10^3 \pm 0.33x10^3$ [a]**	$1.8x10^2 \pm 87$ [b]	1.1 ± 0 [c]
(4S)-4-hydroxy-4-methyloxan-2-one (mevalonolactone)	2	**$4.82x10^2 \pm 16$ [a]**	$1.51x10^2 \pm 7$ [b]	+
2-Hydroxypropane-1,2,3-tricarboxylic acid (citric acid)	1	**$3.25x10^4 \pm 0.5x10^4$ [a]**	$1.45x10^4 \pm 0.13x10^4$ [b]	$2.51x10^3 \pm 30$ [c]
Benzoic acid	1	4 ± 1 [c]	**$1.54x10^2 \pm 5$ [a]**	17.1 ± 0.2 [b]
2-Hydroxybenzoic acid (salicylic acid)	2	0.5 ± 0 [a]	0.6 ± 0.1 [a]	+
3-Phenylpropanoic acid (hydrocinnamic acid)	2	+	+	+
3-(4-hydroxyphenyl)propanoic acid	2	**$4.95x10^2 \pm 22$ [a]**	13 ± 0.4 [b]	-
(S)-2-Hydroxy-3-phenylpropanoic acid (phenyllactic acid)	2	**$5.96x10^2 \pm 24$ [a]**	12 ± 1 [b]	12 ± 0.1 [b]
2-Hydroxy-3-(4-hydroxyphenyl)propanoic acid (hydroxyphenyllactic acid)	2	**$3.12x10^2 \pm 12.5$ [a]**	11 ± 1 [b]	-
Nonanedioic acid (azelaic acid)	2	-	+	**0.5 ± 0.1**
Fatty acids				
Butyric acid	3	8.7 ± 2.44 [b]	6.6 ± 0.70 [b]	**$4.45x10^2 \pm 0.94$ [a]**
Caproic acid	3	1.2 ± 0.51 [b]	1.4 ± 0.40 [b]	**6.3 ± 0.22 [a]**
Heptanoic acid	3	-	**0.1 ± 0.20**	-

(S)-2-Hydroxy-4-methylpentanoic acid ((S)-(-)-2-hydroxyisocaproic acid)	2	**$5.37x10^2 \pm 30$** [a]	$2.22x10^2 \pm 8$ [b]	0.7 ± 0.1 [c]
Caprylic acid	3	0.8±0.75 [a]	**1.4±0.10** [a]	-
Nonanoic acid	3	1.1±0.09 [b]	**1.8±0.30** [a]	1.4±0.09 [a]
Capric acid	3	1.6±0.50 [c]	3.0±0.30 [b]	**4.5±0.25** [a]
3-Hydroxydecanoic acid	2	+	+	+
Lauric acid	3	2.7±0.17 c	**5.0±0.20** [a]	3.1±0.01 [b]
2-Hydroxydodecanoic acid (2-hydroxylauric acid)	2	-	+	-
3-Hydroxydodecanoic acid (3-hydroxylauric acid)	2	+	+	+
Tridecanoic acid	3	**1.5±0.20** [a]	0.3±0.30 [b]	-
Myristic acid	3	2.8±1.05 [b]	9.7±0.80 [a]	**11.0±6.69** [ab]
Pentadecanoic acid	3	1.8±0.67 [a]	1.1±0.10 [a]	**4.1±2.78** [a]
Palmitic acid	3	21.0±5.65 [b]	**49.9±5.60** [a]	31.9±15.94 [ab]
Palmitoleic acid	3	0.9±0.89 [a]	1.6±1.00 [a]	**6.6±4.85** [a]
Stearic acid	3	13.2±5.02 [b]	**32.0±3.40** [a]	8.5±3.13 [b]
Oleic acid	3	0.6±1.00 [b]	**8.8±1.30** [a]	5.8±2.52 [a]
Linoleic acid	3	**0.3±0.46**	-	-
Nonadecanoic acid	3	**0.9±1.48**	-	-
Arachidic acid	3	**1.7±2.88**	-	-

Results are expressed as the mean of three replicates ± standard deviation. Within a same line, means with different letters are significantly different according to a Fisher's least significant difference test ($p < 0.05$).

Legend: (+) value between LOD and LOQ; (-) molecule not detected or at concentration < LOD. Bold values correspond to the highest concentration among the three analyzed fermentates (bold characters correspond to the highest observed values). Identification and quantification method: (1) HPLC coupled to UV or refractometer detectors; (2) LC–Q–ToF; (3) GC–FID.

Table 2. Peptides identified in the studied fermentates and tested for their antifungal activity

Peptide	Amino acid sequence	Length	Charge	Protein	Fermentate
pepa4c177	RLNFLKKIS	9	3	α_{s2}-casein f(165-203)	*L. rhamnosus*
pepa4c223	YLKTVYQHQ	9	2	α_{s2}-casein f(165-203)	*L. rhamnosus*
pepa5b99	QKPVALINNQFLPYPYYAKPA	21	1	κ-casein f(43-97)	*L. rhamnosus*
pepa5b83	PYYAKPAAVRSPAQILQWQVL	21	2	κ-casein f(43-97)	*L. rhamnosus*
pepa5c104	QQKPVALINNQFLPYPYYAKPA	22	1	κ-casein f(43-97)	*L. rhamnosus*
pepa5c165	YYQQKPVALINNQFLPYPYYAKPAA	25	2	κ-casein f(43-97)	*L. rhamnosus*
pepa4b78	KLTEEEKNR	9	0	α_{s2}-casein f(165-203)	*M. lanceolatus*
pepac192	TKLTEEEKNR	10	0	α_{s2}-casein f(165-203)	*M. lanceolatus*
pepa4b97	KTKLTEEEKNR	11	1	α_{s2}-casein f(165-203)	*M. lanceolatus*
pepa4b61	LTEEEKNRLNF	11	-1	α_{s2}-casein f(165-203)	*M. lanceolatus*
pepa4b98	KTKLTEEEKNRL	12	1	α_{s2}-casein f(165-203)	*M. lanceolatus*
pepa4c197	TKKTKLTEEEKNR	13	2	α_{s2}-casein f(165-203)	*M. lanceolatus*
pepa4c190	STEVFTKKTKLTEEEKNR	18	1	α_{s2}-casein f(165-203	*M. lanceolatus*
pepa4b79	KLTEEEKNRLNFL	13	0	α_{s2}-casein f(165-203)	*P. jensenii*
pepa4c101	KTKLTEEEKNRLNFLK	16	2	α_{s2}-casein f(165-203)	*P. jensenii*
pepa5b48	LINNQFLPYPYYAKPA	16	1	κ-casein f(43-97)	*P. jensenii*

Figure legends.

Figure 1. Mean-centered normalized heatmap showing the main volatile compounds present in the studied antifungal fermentates (relative abundance of each compound is shown in brackets).

Figure 2. Antifungal activity of peptide pepa4c177 on *Rhodotorula mucilaginosa* (A) and *Mucor racemosus* (B) grown in potato dextrose broth supplemented with 1% agar at 25°C for 7 days. Five concentrations were tested 0.25, 0.5, 1.0, 2.0 and 5 mg/mL. Central wells were filled with sterile water as negative control.

Figure 1.

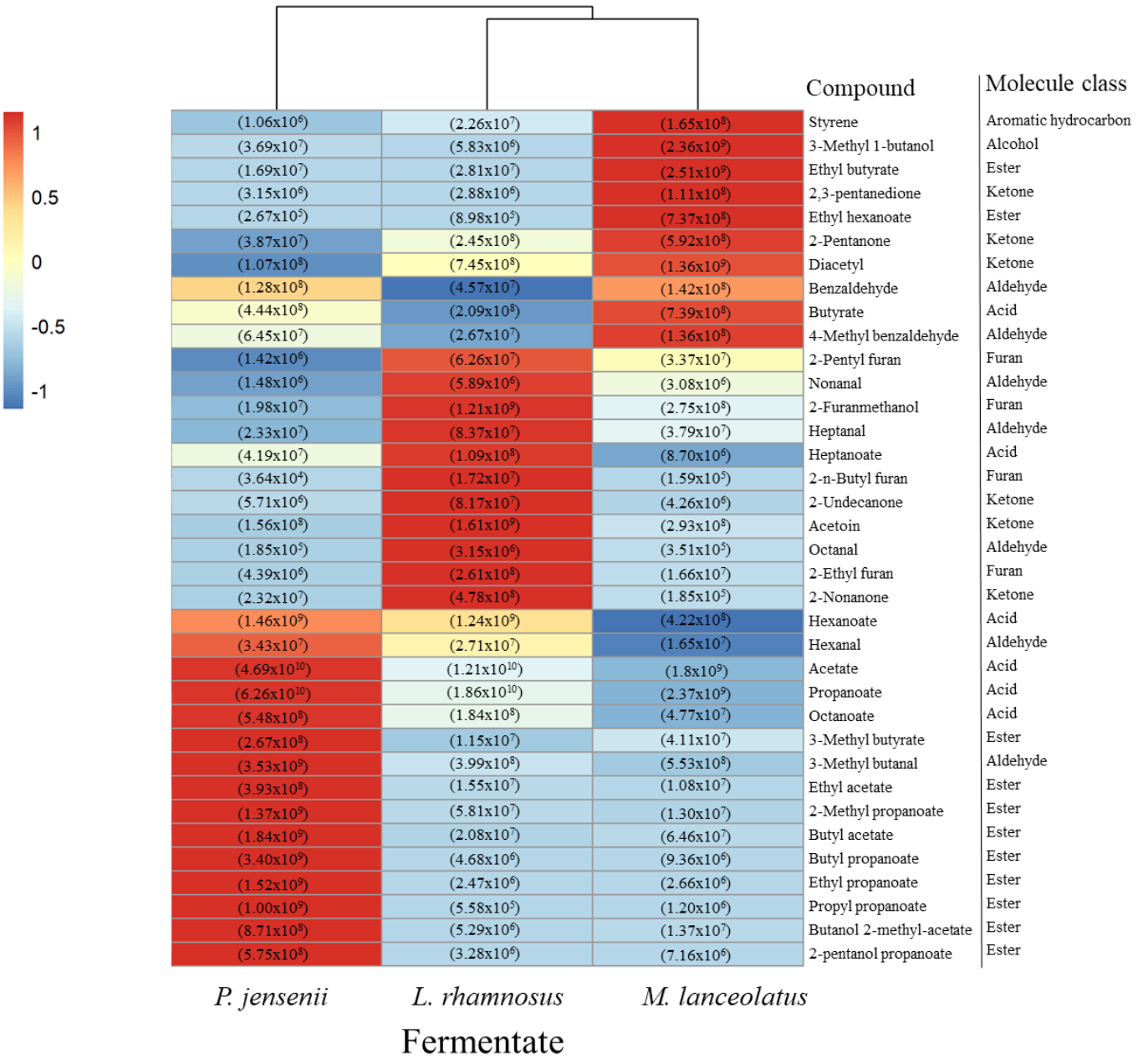
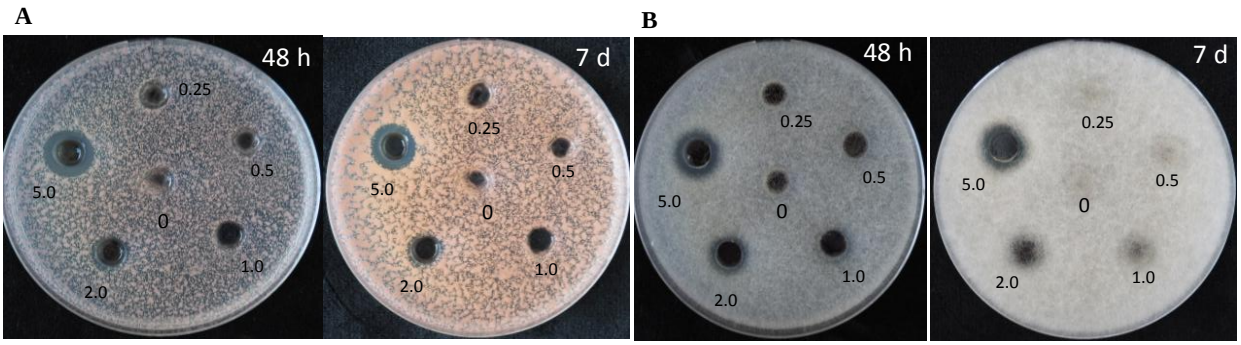

1
0.5
0
-0.5
-1
Compound
Molecule class
(1.06x10^6) (2.26x10^7) (1.65x10^8) Styrene Aromatic hydrocarbon
(3.69x10^7) (5.83x10^6) (2.36x10^9) 3-Methyl 1-butanol Alcohol
(1.69x10^7) (2.81x10^7) (2.51x10^9) Ethyl butyrate Ester
(3.15x10^6) (2.88x10^6) (1.11x10^8) 2,3-pentanedione Ketone
(2.67x10^5) (8.98x10^5) (7.37x10^8) Ethyl hexanoate Ester
(3.87x10^7) (2.45x10^8) (5.92x10^8) 2-Pentanone Ketone
(1.07x10^8) (7.45x10^8) (1.36x10^9) Diacetyl Ketone
(1.28x10^8) (4.57x10^7) (1.42x10^8) Benzaldehyde Aldehyde
(4.44x10^8) (2.09x10^8) (7.39x10^8) Butyrate Acid
(6.45x10^7) (2.67x10^7) (1.36x10^8) 4-Methyl benzaldehyde Aldehyde
(1.42x10^6) (6.26x10^7) (3.37x10^7) 2-Pentyl furan Furan
(1.48x10^6) (5.89x10^6) (3.08x10^6) Nonanal Aldehyde
(1.98x10^7) (1.21x10^9) (2.75x10^8) 2-Furanmethanol Furan
(2.33x10^7) (8.37x10^7) (3.79x10^7) Heptanal Aldehyde
(4.19x10^7) (1.09x10^8) (8.70x10^6) Heptanoate Acid
(3.64x10^4) (1.72x10^7) (1.59x10^5) 2-n-Butyl furan Furan
(5.71x10^6) (8.17x10^7) (4.26x10^6) 2-Undecanone Ketone
(1.56x10^8) (1.61x10^9) (2.93x10^8) Acetoin Ketone
(1.85x10^5) (3.15x10^6) (3.51x10^5) Octanal Aldehyde
(4.39x10^6) (2.61x10^8) (1.66x10^7) 2-Ethyl furan Furan
(2.32x10^7) (4.78x10^8) (1.85x10^5) 2-Nonanone Ketone
(1.46x10^9) (1.24x10^9) (4.22x10^8) Hexanoate Acid
(3.43x10^7) (2.71x10^7) (1.65x10^7) Hexanal Aldehyde
(4.69x10^10) (1.21x10^10) (1.8x10^9) Acetate Acid
(6.26x10^10) (1.86x10^10) (2.37x10^9) Propanoate Acid
(5.48x10^8) (1.84x10^8) (4.77x10^7) Octanoate Acid
(2.67x10^8) (1.15x10^7) (4.11x10^7) 3-Methyl butyrate Ester
(3.53x10^9) (3.99x10^8) (5.53x10^8) 3-Methyl butanal Aldehyde
(3.93x10^8) (1.55x10^7) (1.08x10^7) Ethyl acetate Ester
(1.37x10^9) (5.81x10^7) (1.30x10^7) 2-Methyl propanoate Ester
(1.84x10^9) (2.08x10^7) (6.46x10^7) Butyl acetate Ester
(3.40x10^9) (4.68x10^6) (9.36x10^6) Butyl propanoate Ester
(1.52x10^9) (2.47x10^6) (2.66x10^6) Ethyl propanoate Ester
(1.00x10^9) (5.58x10^5) (1.20x10^6) Propyl propanoate Ester
(8.71x10^8) (5.29x10^6) (1.37x10^7) Butanol 2-methyl-acetate Ester
(5.75x10^8) (3.28x10^6) (7.16x10^6) 2-pentanol propanoate Ester
P. jensenii
L. rhamnosus
M. lanceolatus
Fermentate

Figure 2.


A
48 h
0.25
0.5
5.0
0
1.0
2.0
7 d
0.25
0.5
5.0
0
1.0
2.0
B
48 h
0.25
0.5
5.0
0
1.0
2.0
7 d
0.25
0.5
5.0
0
1.0
2.0